TITLE OF THE INVENTION

CHROMATOGRAPHIC METHOD FOR RNA STABILIZATION

CROSS REFERENCE TO RELATED CO-PENDING APPLICATIONS

This application claims priority from the following co-pending, commonly assigned provisional applications, each filed under 35 U.S.C. §111(b):

U.S. Provisional Application No. 60/187,974, filed March 9, 2000; and

U.S. Provisional Application No. 60/213,948, filed June 23, 2000,

both of which are incorporated by reference herein in their entirey.

FIELD OF THE INVENTION

The present invention is directed to the analysis of RNA molecules by liquid chromatography. More specifically, the invention is directed to a liquid chromatography system and method, such as Matched Ion Polynucleotide Chromatography, which enhances the purification and stabilization of RNA. This invention is directed to methods and systems for inhibiting RNase mediated degradation of RNA. In particular, the invention concerns chromatographic methods for removing RNase from solutions containing RNA.

BACKGROUND OF THE INVENTION

RNA molecules are polymers comprising sub-units called ribonucleotides. The four ribonucleotides found in RNA comprise a common cyclic sugar, ribose, which is covalently bonded to any of the four bases, adenine (a purine), guanine (a purine), cytosine (a pyrimidine), and uracil (a pyrimidine), referred to herein as A, G, C, and U respectively. A variety of modified bases are also encountered in RNA. A phosphate group links a 3'-hydroxyl of one ribonucleotide with the 5'-hydroxyl of another ribonucleotide to form a polymeric chain. Secondary structure commonly occurs via intra-chain hydrogen bonds between complementary bases.

Ribonucleic acid (RNA) transports genetic information within the cell. In the most general terms, RNA passes specific peptide and protein coding information from the genome in the nucleus to those parts of the cell responsible

for the production of these peptides and proteins. There are a number of different RNAs found in the cell at any given time. Some of them are noted below, along with their functions:

Messenger RNA (mRNA): Carries the coding messages to the ribosomes for the production of peptides and proteins;

Small nuclear RNA (snRNA): Responsible for removing intronic sequences from mRNA precursors (preribosomal mRNAs), prior to the spliced mRNA delivery to the ribosomes;

Transfer RNA (tRNA): Provide amino acids "adaptors" to the mRNA codes in the process of protein synthesis;

Ribosomal RNA (rRNA): RNA within the ribosomes themselves, which by association, are part of the protein synthesis process.

Within molecular biology, it is often necessary to isolate these RNA molecules, particularly mRNA. This is because an mRNA "message" indicates that a gene has been transcribed. Furthermore, the extent to which the gene is expressed (up-regulated, down-regulated, turned on, turned off) is often proportional to the amount of gene-specific RNA (mRNA) present in the cell. The quantitation of gene expression via mRNA content can occur by various means (e.g., RT-PCR, expression array/hybridization analysis, etc.)

On other occasions it is desirable to create a compilation, or "library", of those genetic messages being expressed in a cell or cells under a given set of conditions (i.e., normal vs. diseased state). This is often performed by selectively harvesting the mRNAs present in a sample, reverse transcribing the mRNAs to cDNA (first strands and second strands), and then cloning these double stranded sequences into some suitable vector. Once cloned, the cDNA "libraries" can be utilized in various procedures.

RNA is thus the starting material in numerous molecular biology experiments involving the identification of unknown genes and assignment of functions to various proteins. Quality, quantity, purity, and size distribution of RNA determine the rate of success in applications such as cDNA library construction, Northern blot analysis, reverse transcription, and in situ analysis.

Present techniques for the purification of RNA are labor intensive and lengthy. Quantification is routinely performed by gel visualization and spectrophotometric techniques. Sizing and quality determination is often performed by electrophoresis on denaturing agarose gels. However, RNA can become covalently modified by the chemicals used during the fractionation process (e.g., formaldehyde). Many of the present separation techniques require the use of hazardous chemicals (e.g., methylmercuric hydroxide).

In the preparation of mRNA from total RNA, spin columns containing beads coated with poly T oligomers are often used (e.g., Poly(A)Pure™ mRNA Purification Kit, Ambion, Inc., Austin, TX; Oligotex™ mRNA Purification System, Qiagen, Inc., Valencia, CA). The disadvantages of this technique include a requirement for high amounts of total RNA sample due to low recovery of mRNA, contamination of the product (e.g. by rRNA), and degradation of the mRNA product.

There is a need for faster, safer, more reliable, less labor intensive, and more accurate methods of RNA analysis.

Separations of polynucleotides such as RNA have been traditionally performed using electrophoresis through agarose gels or sedimentation through sucrose gradients. However, liquid chromatographic analysis of polynucleotides is becoming more important because of the ability to automate the process and to collect fractions.

Traditional chromatography is a separation process based on partitioning of mixture components between a "stationary phase" and a "mobile phase". The stationary phase is provided by the surface of solid materials which can comprise many different materials in the form of particles or passageway surfaces of cellulose, silica gel, coated silica gel, polymer beads, polysaccharides, and the like. These materials can be supported on solid surfaces such as on glass plates or packed in a column. The mobile phase can be a liquid or a gas in gas chromatography.

The separation principles are generally the same regardless of the materials used, the form of the materials, or the apparatus used. The different

components of a mixture have different respective degrees of solubility in the stationary phase and in the mobile phase. Therefore, as the mobile phase flows over the stationary phase, there is an equilibrium in which the sample components are partitioned between the stationary phase and the mobile phase. As the mobile phase passes through the column, the equilibrium is constantly shifted in favor of the mobile phase. This occurs because the equilibrium mixture, at any time, sees fresh mobile phase and partitions into the fresh mobile phase. As the mobile phase is carried down the column, the mobile phase sees fresh stationary phase and partitions into the stationary phase. Eventually, at the end of the column, there is no more stationary phase and the sample simply leaves the column in the mobile phase.

A separation of a mixture of components occurs because the mixture components have slightly different affinities for the stationary phase and/or solubilities in the mobile phase, and therefore have different partition equilibrium values. Therefore, the mixture components pass down the column at different rates.

In traditional liquid chromatography, a glass column is packed with stationary phase particles and mobile phase passes through the column, pulled only by gravity. However, when smaller stationary phase particles are used in the column, the pull of gravity alone is insufficient to cause the mobile phase to flow through the column. Instead, pressure must be applied. However, glass columns can only withstand about 200psi. Passing a mobile phase through a column packed with 5 micron particles requires a pressure of about 2000 psi or more to be applied to the column. 5 to 10 micron particles are standard today. Particles smaller than 5 microns are used for especially difficult separations or certain special cases). This process is denoted by the term "high pressure liquid chromatography" or HPLC.

HPLC has enabled the use of a far greater variety of types of particles used to separate a greater variety of chemical structures than was possible with large particle gravity columns. The separation principle, however, is still the same.

An HPLC-based ion pairing chromatographic method was recently introduced to effectively separate mixtures of polynucleotides wherein the separations are based on base pair length (U.S. Patent No. 5,585,236 to Bonn (1996); Huber, et al., *Chromatographia* 37:653 (1993); Huber, et al., *Anal. Biochem.* 212:351 (1993)). Ion pair reverse phase high pressure liquid chromatography (IPRPHPLC) was used as a process for separating DNA using non-polar separation media, wherein the process used a counterion agent, and an organic solvent to release the DNA from the separation media. This method was used in the separation of double stranded DNA of up to about 1,000 base pairs and for the separation of single stranded DNA of up to about 100 nucleotides.

Many applications in molecular biology require the use of intact RNA. Ribonucleases (RNases) are extremely difficult to inactivate. Great care must be taken to avoid inadvertently introducing RNases into RNA preparations both during and after isolation. This is especially important if the starting material is difficult to obtain or is irreplaceable. Common RNases include RNase A, B, C, 1 and T1.

Thus, work in molecular biology and recombinant nucleic acid technology has been encumbered by problems associated with degradation of RNA by ribonucleases. Researchers in the prior art have relied on protein-based RNase inhibitors (e.g., RNASIN (Promega Corp., Madison, WI); SUPERASE-IN (Ambion, Inc., Austin, TX)), or the use of alkylating reagents such as diethylpyrocarbonate to inhibit the activity of ribonucleases. However, protein-based inhibitors often require lengthy incubation procedures. Alkylating reagents are toxic or carcinogenic chemicals. Some of the chemicals routinely used in purifying RNA produce undesirable covalent adducts with RNA.

In addition, in order to inhibit RNase activity, RNA containing samples are routinely stored at -70°C which can be inconvenient and costly.

There is a need, therefore, for improved methods and systems for preventing degradation of RNA.

SUMMARY OF THE INVENTION

Objects of the present invention include providing a method and system for segregating RNA molecules that is fast, safe, reliable, convenient, reproducible, and quantitative. Other objects of the present invention include providing methods and systems for stabilizing RNA which are convenient, rapid, and which minimizes use of toxic and reactive chemicals.

One aspect of the invention provides a method for stabilizing an RNA molecule against degradation that comprises applying the RNA molecule to a separation medium having a non-polar separation surface in the presence of a counterion agent; eluting the RNA molecule from the separation medium by passing through the separation medium a mobile phase containing a concentration of organic solvent sufficient to elute the RNA molecule from the separation medium; and collecting an eluant fraction containing the RNA molecule, wherein the RNA molecule is stabilized against degradation.

In a preferred embodiment, the invention provides a method for stabilizing an RNA molecule against degradation that comprises applying a solution to a separation medium having a non-polar separation surface in the presence of a counterion agent, wherein the solution comprises the RNA molecule and an agent capable of catalyzing the degradation of RNA; eluting the RNA molecule from the separation medium by passing through the separation medium a mobile phase containing a concentration of organic solvent sufficient to elute the RNA molecule from the separation medium, where the elution is conducted under conditions that result in a substantial separation of the RNA molecule from the agent capable of catalyzing the degradation of RNA; and collecting an eluant fraction containing the RNA molecule that is substantially free of the agent capable of catalyzing the degradation of RNA.

In a preferred embodiment of the invention the agent capable of catalyzing the degradation of RNA is an enzyme, especially an RNase (e.g., RNase 1, RNase A).

The invention is capable of stabilizing a plurality of RNA molecules.

In a preferred embodiment of the invention MIPC is used to separate the RNA molecule from the agent capable of catalyzing RNA degradation. Alternatively, the RNA molecule can be separated from the agent capable of catalyzing RNA degradation in a batch process.

In another preferred embodiment of the invention, the RNA molecule is separated from the agent capable of catalyzing RNA degradation under conditions wherein the secondary structure of the RNA molecule is substantially denatured. This is preferably accomplished by separating the RNA molecule from the agent capable of catalyzing RNA degradation at a temperature of about 50°C or greater, more preferably at a temperature of about 70°C or greater. Alternatively, the mRNA molecule can be substantially denatured by means of a chemical reagent.

In another preferred embodiment of the invention, the separation is conducted under conditions that are substantially free of multivalent cations capable of interfering with polynucleotide separations.

In yet another preferred embodiment of the invention, the separation medium comprises particles selected from the group consisting of silica, silica carbide, silica nitrite, titanium oxide, aluminum oxide, zirconium oxide, carbon, insoluble polysaccharide, and diatomaceous earth, the particles having separation surfaces which are coated with a hydrocarbon or non-polar hydrocarbon substituted polymer, or have substantially all polar groups reacted with a non-polar hydrocarbon or substituted hydrocarbon group, wherein the surfaces are non-polar.

In yet another preferred embodiment of the invention, the separation medium comprises polymer beads having an average diameter of 0.5 to 100 microns, the beads being unsubstituted polymer beads or polymer beads substituted with a moiety selected from the group consisting of hydrocarbon having from one to 1,000,000 carbons, especially a separation medium comprising C-18 alkylated nonporous poly(styrene-divinylbenzene) polymer beads.

In yet another preferred embodiment of the invention, the separation medium comprises a monolith.

In yet another preferred embodiment of the invention, wherein the separation medium is substantially free of multivalent cations capable of interfering with polynucleotide separations.

The separation medium is preferably prepared using reagents that are substantially free of multivalent cations capable of interfering with polynucleotide separations and under conditions that are substantially free of multivalent cations capable of interfering with polynucleotide separations. It is also desirable to subject the separation medium to acid wash treatment to remove any residual surface metal contaminants and/or to treatment with a multivalent cation-binding agent.

In another preferred embodiment of the invention, the mobile phase includes an organic solvent selected from the group consisting of alcohol, nitrile, dimethylformamide, tetrahydrofuran, ester, ether, and mixtures of one or more thereof. Acetonitrile is a particularly preferred organic solvent in this regard.

In yet another preferred embodiment of the invention, the mobile phase includes a counterion agent selected from the group consisting of lower alkyl primary amine, lower alkyl secondary amine, lower alkyl tertiary amine, lower trialkylammonium salt, quaternary ammonium salt, and mixtures of one or more thereof. Particularly preferred counterions include octylammonium acetate, octadimethylammonium acetate, decylammonium acetate, octadecylammonium acetate, pyridiniumammonium acetate, cyclohexylammonium acetate, diethylammonium acetate, propylethylammonium acetate, propyldiethylammonium acetate, butylethylammonium acetate, methylhexylammonium acetate, tetramethylammonium acetate, tetraethylammonium acetate, tetrapropylammonium acetate, tetrabutylammonium acetate, dimethydiethylammonium acetate, triethylammonium acetate, tripropylammonium acetate, tributylammonium acetate, tetrapropylammonium acetate, tetrabutylammonium acetate, triethylammonium hexafluoroisopropyl alcohol, and mixtures of one or more

thereof. Particularly preferred counterion agents include tetrabutylammonium bromide and triethylammonium acetate.

In especially preferred embodiments of the invention the RNA molecule is separated from the agent capable of catalyzing RNA degradation by MIPC, wherein mRNA denaturation is achieved by conducting the separation at a temperature sufficient to substantially denature the mRNA molecule, wherein the separation medium comprises polymer beads having an average diameter of 0.5 to 100 microns, and wherein the mobile phase comprises acetonitrile and triethylammonium acetate. In the most preferred embodiment of the invention, the separation is conducted under conditions that are substantially free of multivalent cations capable of interfering with polynucleotide separations and at a temperature of about 70°C or greater, and the separation medium comprises C-18 alkylated nonporous poly(styrene-divinylbenzene) polymer beads.

In another aspect, the invention provides a stabilized RNA molecule prepared by the process recited above

In yet another aspect, the invention provides a solution of RNA molecules that is substantially free of RNases.

In still another aspect, the invention provides a stabilized solution of RNA molecules that is devoid of RNAse inhibitors and stable at room temperature.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of a single column MIPC system using valves and valve controls to establish mobile phase gradients.

FIG. 2 is a partial schematic representation of a pump system for establishing mobile phase gradients.

FIG. 3 is a schematic representation of an autosampler subsystem.

FIG. 4 is a schematic representation of an injection valve used in the MIPC system.

FIG. 5 is a schematic representation of an injection valve in the filled loop load position.

FIG. 6 is a schematic representation of an injection valve in the filled loop injection position.

FIG. 7 is a schematic representation of an injection valve in the partial loop load position.

FIG. 8 is a schematic representation of the injection valve in the partial loop injection position.

FIG. 9 is a front view of the separation compartment of an MIPC column oven.

FIG. 10 is a top view of the HPLC DNA analyzer column oven shown in FIG. 9.

FIG. 11 is an end view of the compact column heater embodiment of this invention.

FIG. 12 is a cross-sectional view taken along the line A—A in FIG. 11.

FIG. 13 is a schematic view of a Peltier heater/cooler embodiment of this invention.

FIG. 14 is a representation of the physical structure of a representative reverse phase chromatographic column.

FIG. 15 is a chromatogram from a MIPC analysis of RNA size markers. Peaks are labeled with the number of nucleotides of the eluted molecules.

FIG. 16 is a chromatogram from a MIPC analysis of total RNA from a plant extract.

FIG. 17 is a chromatogram from a MIPC analysis of RNA from a plant extract after a first affinity purification.

FIG. 18 is a chromatogram from a MIPC analysis of RNA from a plant extract after a second affinity purification.

FIG. 19 is a chromatogram from a MIPC analysis of mouse brain mRNA.

FIG. 20 is a chromatogram from a MIPC analysis of human brain mRNA.

FIG. 21 is a chromatogram from a MIPC analysis of human brain mRNA.

FIG. 22 illustrates an MIPC elution profile for a mouse brain mRNA preparation.

FIG. 23 is a chromatogram from a MIPC analysis of RNA size markers.

FIG. 24 illustrates an MIPC elution profile of a RT/PCR cDNA product prepared from a fraction collected during MIPC elution of FIG. 19.

FIG. 25 illustrates an MIPC chromatograph after the injection of a water sample.

FIG. 26 illustrates an MIPC chromatograph after the injection of a solution containing RNase 1.

FIG. 27 illustrates the effect of acetonitrile concentration on the stability of mRNA stored in solution.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the present invention concerns a Matched Ion Polynucleotide Chromatography (MIPC) method and system for segregating a mixture of RNA molecules.

"Matched Ion Polynucleotide Chromatography" as defined herein, includes a process for segregating RNA moleucles using non-polar reverse phase media, wherein the process uses a counterion agent, and an organic solvent to release the polynucleotides from the reverse phase media.

"Segregating" as defined herein includes a Matched Ion Polynucleotide Chromatography process for separating RNA molecules in which the retention time of a molecule is primarily based on nucleotide length but in which the retention time can be subject to bias due to the influence of the polarity of the bases. The bias can either increase or decrease the retention time.

MIPC process are described in earlier, copending and commonly assigned U.S. Patents or Patent Applications U.S. Patent Nos. 5,772,889; 5,997,742; 5,972,222; 5,986,085; 6,017,457; U.S. Patent Application Nos. 09/058,580 filed April 10, 1998; 09/183,123 filed October 30, 1998; 09/183,450 filed October 30, 1998; 09/350,737 filed July 9, 1999; 09/318,407 filed May 25, 1999; 09/469,551 filed December 22, 1999, each of which is incorporated by reference in its entirety herein.

The preferred MIPC system provides automated options for sample selection, mobile phase gradient selection and control, column and mobile phase temperature control, and fraction collection.

FIG. 1 is a schematic layout of the system in accordance with one embodiment of the MIPC system. A plurality of containers can be used as

reservoirs for solutions, such as solvents, counterions, and other solutions, which make up the mobile phase. For example, container 2 can contain an aqueous component of a mobile phase such as an aqueous solution of counterion agent (e.g., triethylammonium acetate (TEAA)), and container 4 can contain an aqueous solution of counterion agent plus organic (driving) solvent (e.g., TEAA plus acetonitrile). An auxiliary liquid (e.g., a co-solvent) can be held in container 6. These solutions are mixed to achieve a selected concentration of organic solvent in the mobile phase during a separation. Other examples of these solutions are provided in the Examples herein and in the commonly assigned patent indicated hereinabove. The containers have respective transport tubing such as counterion solution transport tubing 8, solvent solution transport tubing 10, and auxillary liquid transport tubing 12 communicating therewith, and leading to degasser 14.

The degasser 14 removes dissolved gases from the liquids. An example of a suitable degasser is the Degassit Model 6324. Removal of dissolved oxygen is particularly important because its presence increases the risk of oxidizing ferrous or other oxidizable metals in the system components and thus introducing the corresponding cations into the mobile phase liquid.

Column cleaning solution is contained in cleaning solution container 16 which likewise has a cleaning solution transport conduit 18 communicating therewith leading to the degasser 14. In this embodiment, the cleaning solution can flow by gravity pressure if the container 16 is elevated above the degasser and injection valve 54. Alternatively, a pump 110 as shown in FIG. 2 can be provided to achieve cleaning solution flow.

The system of the invention incorporates conventional mobile phase flow control means which controls flow of solvent solution and aqueous components of a mobile phase. In one embodiment, the mobile phase flow control means comprises a set of flow control valves, each with automatic opening controls under computer control as described hereinbelow. In another embodiment the mobile phase flow control means comprises a set of pumps, the flow setting of which are responsive to computer control as described hereinbelow

The system illustrated in FIG. 1 utilizes one embodiment of a mobile phase flow control means which includes a set of flow control valves. Degassed counterion solution conduit 20, degassed solvent solution conduit 22, and degassed auxiliary liquid conduit 24 leading from the degasser 14 communicate with respective aqueous component proportioning valve 26, solvent solution proportioning valve 28, and auxiliary liquid proportioning valve 30. The settings for these proportioning valves are set and changed by valve operators such as stepper motors associated therewith, and these valve operators respond to establish a desired set of settings in response to commands from the mobile phase flow control software module described in greater detail hereinbelow. The flow control valves 26, 28, and 30 comprise an embodiment of a mobile phase flow control means which controls the flow of solvent solution and other components of the mobile phase. The settings for these valves control the ratio of liquids (co-solvents, solvent solution, etc.) through the injector valve and the separation column. Conduits 32, 34, and 36 lead from respective proportioning valves 26, 28 and 30 to the intake of pump 38.

The cleaning solution transport conduit 31 leads to a cleaning solution valve 40. An optional cleaning solution conduit 42 leads from the valve 40 and communicates with the inlet of pump 38. Valve 33 controls flow through conduit 42.

The openings of valves 26, 28 and 30 accurately set the relative ratios of the organic solvent, and other components, within the mobile phase, a most important part of this system because the RNA segregation by MIPC is a function of solvent concentration. As will be described in regard to the various RNA segregation processes, the slope of the organic solvent gradient as a function of time is changed during the separation process, and the most critical phase may require a very precise gradient. The settings of the valves 26, 28 and 30 are established by conventional valve actuators which can be remotely set by signals to a conventional valve control device.

In a preferred embodiment, the separation system is under computer control as represented at 35. The computer includes Instrument Control

Software which provides computer controlled instructions for establishing the settings of valves 26, 28 and 30 to precise flow values at appropriate times during the operation of the system.

In a similar manner, the Instrument Control Software of the instant invention provides computer controlled instructions to establish the operational parameters of the pump 38, such as the off/on status of the pump and the pressure or flow rate settings of the pump.

Pump outflow conduit 44 communicates with the in-line mixer 46, directing the liquid flow through the mixer 46 for thorough mixing of the components. Mixed liquid outflow conduit 48 communicates with optional guard column 50 to treat the mixed liquid to remove multivalent metal cations and other contaminants which would interfere with the separation of RNA molecules. Guard column 50 can contain a cation exchange resin in sodium or hydrogen form for removal of multivalent metal cations by conventional ion exchange. Conduit 52 communicates with the outlet of the guard column and an inlet port of a cleaning solution injector valve 54. Cleaning solution supply conduit 56 connects valve 40 with the cleaning solution injector valve 54, and waste outlet conduit 58 leads to waste. Conduit 60 leads from valve 54 to the sample injection valve 62.

Sample aliquot selector 64 communicates with injector valve 62 through sample conduit 66. Waste conduit 68 leads from the injector valve and removes waste liquids.

In the injector valve 62, the sample is introduced into a stream of solvent and carrier liquid passing through the valve from conduit 60. Sample conduit 70 communicates with an outlet port of injector valve 62 and with the column prefilter 74 in the air bath oven 72. The capillary tubing coil 76 communicates with the prefilter 74 and the inlet of chromatography column 78. The extended length of the capillary coil 76 allows ample heat to pass from the heated oven air into the liquid passing through the coil, bringing the liquid within $\pm$ 0.05°C of a selected temperature. The oven 72 establishes this temperature uniformity in the prefilter 74, coil 76, and chromatography column 78.

The separation column 78 is packed with beads having a unique separation surface which effects segregation of RNA molecules in the presence of a counterion by the MICP process. The separation process and details about the column and beads are described in detail hereinbelow. A stream of mobile phase containing segregated RNA molecules passes from the chromatography column 78 through conduit 80.

Conduit 80 communicates with a detector 84. The detector can be a conventional UV absorbance device which measures the UV absorbance of the RNA fragment structures in the liquid mobile phase. The absorbance is a function of the concentration of the RNA fragments in the liquid being tested.

Alternatively, if the RNA is labeled with a fluorescent marker, the detector can be a fluorescence detector which can continuously measure the level of the fluorescent marker in the liquid by detecting the emission level at the frequency most appropriate for the marker. It will be readily apparent that any detecting system capable of continuously measuring a characteristic of the liquid which is a function of the concentration of the RNA molecules therein is suitable and intended to be within the scope of this invention. Examples of suitable detectors include the L-7420 UV-Vis detector, and the L-7480 Fluorescence detector available from Hitachi. The electrical output from the detector preferably is converted to a digital form by an A/D converter and recorded in standard digital format to a digital storage device such as a disk drive in computer 35. Conduit 86 removes the tested liquid.

Then, the mobile phase passes to the automated fraction collector 88 where selected portions of the mobile phase fractions are collected in vials for later processing or analysis. Uncollected fractions are removed through conduit 90.

In the above description, the liquid flow system is described as a series of conduits. The conduits are capillary tubing selected to avoid introduction of multivalent cations into the liquids. The preferred capillary tubing materials are titanium and PEEK. The other components of the system are preferably made of titanium or PEEK or have the surfaces exposed to the liquid coated with PEEK to

protect them from oxidation and prevent the introduction of multivalent cations into the liquid. Stainless steel can also be used but is preferably treated to remove all oxidized surface materials and the solutions contacting the stainless steel surfaces are free of dissolved oxygen.

Illustrating another embodiment of a mobile phase flow control means, FIG. 2 is a partial schematic representation of a pump system for establishing mobile phase composition. This system relies on proportioning pumps to control the ratio of aqueous component and solvent solution, such as solutions A and B described hereinabove. The inlets of proportioning pumps 92, 94 and 96 by way of their respective supply conduits 98, 100, and 102 communicate with the degasser 14, and by way of their respective outlet conduits 104, 106 and 108 communicate with the inline mixer 46. The operational speed for these proportioning pumps are calibrated to flow rates therethrough and are controlled by a flow control software module described in greater detail hereinbelow. The settings for these proportioning valves control the liquid flow speed and the ratio of liquids (co-solvents, driving solvents, etc.) through the injector valve and the separation column.

A pump 110 can supply cleaning solution to the system through optional conduit 112. An optional conduit 107 leads from conduit 112 and communicates with the in-line mixer 46. Valve 111 controls flow through conduit 107.

Examples of suitable mobile phase control means for use in the invention include the programmable dual piston pump Model L-7100 available from Hitachi and the Model 2690 Separations Module available from Waters.

FIG. 3 is a schematic representation of an autosampler subsystem used in the MIPC system. This autosampler removes an aliquot having a predetermined volume from a selected well or vial (e.g., micro-centrifuge tube) supported in a multi-well 113. Microwell plates can have any predetermined number of wells 114 having a precise dimensional position for each well, such as the standard 96 well multiwell plate. The sampling needle 115 is supported on a sampling carriage 116. The sampling carriage 116 has a needle support 118 mounted for vertical movement on vertical support 117. Vertical support 117 is mounted for

lateral movement on carriage 116. Lateral movement of the support 117 positions the needle above a selected well or the injector port 119 of injection valve 120. The flexible tubing 123 is mounted in sealed engagement with the needle 115 at one end and with the syringe needle 124 at the other end. The syringe needle 124 communicates with the inner volume of the syringe cylinder 125. The piston 126 is mounted on the syringe actuator rod 128 and forms a sealed engagement with the inner wall of the cylinder 125. In operation, vertical upward movement of the syringe actuator rod 128 displaces liquid in the cylinder 125, and vertical downward movement of the syringe actuator rod 128 pulls liquid into the syringe. Rod 128 is attached to clamp 130 which is supported for movement along guide element 132. When valve 122 is positioned to provide communication between the needle 124 and the tubing 123, the downward movement of the piston 126 pulls sample into the needle 115 from a well 114. When needle 115 is positioned above injector valve port 119, upward movement of the piston 126 discharges sample from needle 115 into port 119.

Conduit 131 extends from valve 122 to the cleaning solution reservoir 121. When valve 122 is in the position providing communication between the needle 124 and the conduit 131, the downward movement of the piston 126 draws cleaning solution into the needle. When the needle 115 is positioned above the injector port 119 and valve 122 is positioned to provide communication between the needle 124 and the conduit 123, upward movement of the piston 126 discharges cleaning solution into the injector port 119. Examples of suitable autosamplers include the HITACHI Model L-7250 Programmable Autosampler and the HTS PAL High Throughput Autosampler (Shimadzu, Columbia, MD).

FIG. 4 is schematic representation showing the structure of the sample injection valve and cleaning solution injection valve for use in the MIPC system. The same valve structure can be used for both the sample injection and cleaning solution injection. The injection valve 150 is a six-port, rotary valve operated by a conventional valve motor such as a stepper motor (not shown). Exemplary valves include the LabPRO valves available from RHEODYNE (Cotati, CA). The valve has six external ports permanently connected to inlet and outlet conduits.

External port 152 is connected with an injection line 154 for receiving a sample to be analyzed. External port 156 is connected with a column supply conduit 158 communicating with the separation column 78 (FIG. 1). External port 160 is connected with an inlet conduit 162 communicating with the outlet of pump 38 (FIG. 1). External port 164 is connected with a waste conduit 166. Opposed outlet ports 168 and 170 communicate with the opposed sample inlet and outlet ends of a sample loop 172. During the injection of cleaning solution, the valve injects a block of cleaning solution into the solvent stream, regenerating and cleaning the separation column and other components downstream of the injection, removing from the surfaces accumulated residues and any residual RNA remaining from prior segregation procedures.

The connections between the external ports and internal passages, and their operation in the cleaning solution injector valve 54 and sample injector valve 62 in FIG. 1 is described in FIGs. 5-8. The description hereinbelow is presented for the sample injection valve 62, but the same relationships and operation apply to the cleaning solution injection valve with the exception of the liquids being injected and their source.

FIGs. 5 and 6 describe the use of the valve for filled loop injection, the mode used when a larger volume of sample (or cleaning solution) is to be injected. FIG. 5 is a schematic representation of an injection valve in the sample load position, and FIG. 6 is a schematic representation of the injection valve in the injection position. In the load position shown in FIG. 5, a first internal passageway 174 of the valve connects the first end 176 of loop 172 with the sample injection line 154, and a second internal passageway 178 connects the second end 180 of loop 172 with the waste conduit 166. A third internal passageway 182 connects the pump outlet conduit 162 with the conduit 158 to the separation column 78. While sample from the injection port 154 is introduced into the sample loop 172 through passageway 174, any surplus or liquid in the loop 172 is expelled to the waste conduit 166 through passageway 178. Simultaneously, mobile phase solutions flow from the pump conduit 162 to the separation column 78 through third conduit 182.

Rotation of the valve in the direction of arrow 150 to the injection position shown in FIG. 6 moves the internal passageways to establish a different set of connections with the inlet and outlet conduits. Passageway 179 connects one end 180 of the loop 172 with the conduit 158 leading to the separation column, and passageway 175 connects the other end 176 of the loop 172 with the inlet conduit 162 leading to the pump. Mobile phase solution from the pump enters passageway 175 and passes through the loop 172, expelling sample solution into the conduit 158 leading to the column and continues to rinse the loop, carrying any residue into the column conduit 158. Meanwhile, passageway 183 connects the sample injection conduit 154 to waste, permitting passage of cleaning solution, if desired, through passageway 183. This procedure provides a reliable injection of a measured volume of sample solution into the conduit leading to the separation column 78 (FIG. 1), the liquid passing through the prefilter 74 and temperature regulating coil 76 before it reaches the separation column.

The system of the invention incorporates oven temperature control means for controlling the temperature of the separation column and the mobile phase entering the column.

FIGs. 9 and 10 illustrate one embodiment of a temperature control means. FIG. 9 is a front view of the process compartment of an HPLC RNA analyzer column oven, and FIG. 10 is a top view of the HPLC RNA analyzer column oven shown in FIG. 9. The process compartment in the embodiment shown in FIGs. 9 and 10 is divided from the heating compartment by back wall 200 in which air exhaust port 202 is positioned. A metal bar 204 enclosing a temperature sensor such as a thermocouple or thermister is positioned in the port 202 to measure the temperature of the air passing through the port. Capillary tubing 206 leads from the sample injector (not shown) to a prefilter 208. Prefilter 208 is an inline filter or guard cartridge, such as described in U.S. Patent No. 5,772,889, which removes contaminants from the incoming liquid. An elongated coil 210 of capillary tubing has an inlet end in communication with prefilter 208 for receiving mobile phase liquid therefrom. The elongated coil 210 has an outlet end communicating with the inlet end 212 of a separation column 214. Separation column 214 preferably

contains MIPC separation media. Outlet tubing 216 leads from the outlet end 218 of the separation column 214 to detector 84 (FIG. 1). Coil 210 is a liquid heating coil made of a RNA compatible, multivalent cation free tubing such as titanium or PEEK. The length and diameter of tubing used is any length which is sufficient to enable liquid mobile phase passing therethrough to reach the equilibrium temperature of air in the processing compartment. A tubing length of from 6 to 400 cm and a tubing ID of from 0.15 to 0.4 mm is usually sufficient. Since the length of tubing 210 does not degrade the separation of components achieved by the system, the length can be selected based on the length required to achieve effective heating of the process liquids.

Referring to FIG. 10, air from the processing compartment 220 passes through the opening 202 in wall 200, through a heater/fan system 222 for temperature adjustment. The adjusted air received by the heating compartment 224 recycles back to the processing compartment 220 along the passageways 226 defined by the spacing between the sidewalls 227 and the outer oven wall 228. The heating coil in the embodiment shown in FIGs. 9 and 10 provides a temperature accuracy to within the range of $\pm$ 0.2 °C and reduces the temperature equilibrium time between temperature settings to below 5 minutes for temperature changes of 5°C and below 2 minutes for temperature changes of up to 1°C.

FIGs. 11 and 12 illustrate another embodiment of a temperature control means. FIG. 11 is an end view of a compact column heater, and FIG. 12 is a cross-sectional view taken along the line A-A in FIG. 11. This embodiment relies on direct metal-to-metal conduction of heat to and from the system components and does not depend upon an air bath to achieve temperature changes and accuracy. This embodiment is shown for a two column system, although it could be used for a single column, if desired. It comprises heat conducting blocks (230,232) having receptacles sized and shaped to receive the system components. Filter cavity or prefilter receptacles (234,236) have inner surfaces which are sized to receive prefilters (238,240) and establish heat transfer contact with the outer surfaces thereof. Separation column receptacles (242,244) have

inner surfaces sized to receive respective separation columns (246,248) and separation column couplers (250) (one is shown in FIG. 12) which connect capillary tubing to the respective separation columns. Receptacles (242,244) are sized and shaped to establish heat transfer contacts between the inner heat transfer surfaces of blocks (230,232) and the separation column components received therein. Capillary coil receptacles 252 (one is shown in FIG. 12) have an inner surface which is shaped to receive a coils of capillary tubing 254 (one is shown in FIG. 12) and to establish heat transfer contact with the outer surface thereof. In the embodiment shown in these figures, receptacles (234,236) and (242,244) can be cylindrical holes with approximately parallel central axes lying in a common plane. It would be readily apparent to a person skilled in the art that other configurations are equally suitable and all configurations are considered to be within the scope of this invention.

Temperature sensor receptacles (256,258) are provided in heat conducting blocks (230,232). Capillary receptacle passageways 260 for receiving connecting tubing 262 in a heat-conducting relationship are also provided in the heating-conducting block (230,232). The capillary coil receptacles 252 are shown in this figure to be cylindrical cavities with their axes perpendicular to the axes of receptacles (234,236) and (242,244). Optionally, a conductive metal cylinder (not shown) can be positioned within the capillary coils in heat conducting contact with the inner surfaces thereof to increase heat transfer area between the metal block heating assembly and the liquid in the coils. A KAPTON resistance heater or other type of heating unit 264 is positioned between and in heat-conducting contact with surfaces 266 and 268 of heating blocks (230,232) to transfer heat to the heat-conducting blocks. Heat sinks (270,272) are positioned in heat-conducting relationship with opposed cooling surfaces (274,276) of the heat conduct blocks (230,232) to remove heat therefrom. Cooling fans 278 and 280 are in a heat removal relationship with the heat sinks 270 and 272 and are activated to accelerate heat removal therefrom.

The heat conducting blocks 230 and 232, and the heat sinks 270 and 272 are made of a material having high heat conductivity such as aluminum or

copper, although they can be made of other heat-conducting solids such as ferrous metals or any other solid material having the requisite heat conductivity. Heat pipes can also be used as heat sinks.

The capillary tubing can be made of PEEK or titanium, although titanium is preferred for maximum heat transfer efficiency. With this improved heat transfer, the capillary coil can have a fully extended length as short as 5 cm although a minimum coil length of 10 cm is preferred. A longer coil of PEEK tubing would be required to achieve the same heat transfer as titanium capillary tubing.

The system shown in FIGs. 11 and 12 comprises two systems in mirror image. It will be readily apparent that for a single column, half the system would be sufficient and is intended to be included within the scope of this invention. The position, alignment and spacing of the receptacles are not a critical feature of this invention. Any alignment and configuration which provides a compact and heat-transfer efficient result is intended to be included within the scope of this invention.

The embodiments shown in FIGs. 11 and 12 provide a compact heater which is more responsive to heater controls, provides rapid changes from one temperature platform to another, and maintains a temperature accuracy within ± 0.5 °C of a set temperature. The heat transfer rate obtained with the metal-to-metal contact between the heating block and the elements being heated is far greater than can be obtained in an air bath system, providing the more rapid response to a changed temperature and greater temperature accuracy. It also allows process liquid temperature adjustment with a shorter capillary tubing coil.

In yet another illustration of a temperature control means, FIG. 13 shows a schematic view of a preferred Peltier heater/cooler embodiment. Heating block 282 is in conductive contact with a Peltier heating element (not shown) for heating or cooling required to reach and maintain a desired temperature. Channel 284 is a prefilter receptor having an inner surface 286 in heat conductive relationship with prefilter 288. Channel 290 is a column and column guard receptor having an inner surface 292 in heat conductive relationship with coupler 294 and end nut elements 296 of separation column 298. Capillary

tubing 300 communicates with the prefilter 288 and the sample and solution sources (not shown). Capillary tubing 302 from the outlet of the separation column 288 communicates with an analyzer 84 (FIG. 1). Capillary tubing 304 connects the outlet end of the prefilter 288 with the coupler 294, which in turn communicates with the separation column 298. Capillary tubing 304 is received in a labyrinth-like configuration of channels in the heating block 282 to provide increased capillary length and surface contact between the capillary tubing 304 and the heating block 282. The configuration of the labyrinth and tubing can be any configuration which provides an adequate capillary length and surface contact, including additional loops and capillary placement of more than one pass per channel. The capillary tubing 304 can be PEEK or titanium, titanium being preferred because of its high heat conductivity. The heating block 282 can be any heat conductive metal. Aluminum or copper are preferred because of their higher heat conductivity, although ferrous metals such as steel can be used. The Peltier heater is controlled with a conventional temperature and control system (not shown) such as the systems used in Peltier thermocyclers. As with the embodiment shown in FIGs. 11 and 12, the temperature accuracy achieved by the Peltier heated block is $\pm$ 0.5°C.

Features of improved air bath oven and solid block heating systems described hereinabove with respect to FIGs. 9-13 are described in greater detail in commonly owned, copending U.S. Patent Application Serial No. 09/295,474 filed April 19, 1999, the entire contents of which are hereby incorporated by reference.

An important aspect of the present invention concerns the cross-sectional dimension of the separation column 78. Fig. 4 is a partially exploded representation of the physical structure of a representative separation column. The column comprises a cylinder or tube 334 with external ferrule 335 on both ends. The tube has internal diameter (ID) as shown at 333 and is filled with separation media 336. A porous frit 338 is held against the upper surface of the separation media by the end fitting 340. The end fitting 340 receives frit 338 and holds the frit against the end of the tube 334. The internally threaded nut 342

receives the externally threaded fitting 340 in a threaded engagement. The fitting 340 has an internally threaded end receptor 346 for receiving a capillary tubing end coupler (not shown).

The material comprising the cylinder can be polymer or metal. Stainless steel tubes suitable for use in the present invention are available commercially, for example from Isolation Technologies Inc. (Hopedale, MA). Examples include stainless steel tubing having ID sizes such as 4.6, 6.5, 7.8, 10.0, 21.2, 30 and 50mm. Columns as large as 500mm to 1m are also available are suitable for use in the present invention. The column preferably includes porous frits 338 (e.g., as manufactured by Mott Corporation, Farmington, CT) inside the fittings on both ends and can include end seals that screw into the fittings (available from Upchurch Scientific, Oak Harbor, WA and/or Isolation Technologies).

The separation media 336 comprises organic polymer materials or inorganic materials having the requisite structure and non-polar surfaces. Suitable materials are described hereinbelow and in copending, commonly assigned patent application Serial No. 09/183,123 the contents of which are hereby incorporated by reference.

In one aspect, the present invention concerns a chromatographic method for segregating a mixture of RNA molecules having lengths exceeding about 100 nucleotides, said method comprising: a) applying a solution of said fragments and counterion reagent to a column containing polymeric beads having non-polar surfaces, wherein said beads have an average diameter of about 1 to about 100 microns; b) eluting said RNA molecules with a mobile phase which includes counterion reagent and an organic component.

In the practice of the method of the invention, a liquid sample containing RNA is injected onto a MIPC chromatography column containing a reverse phase support. In MIPC, the RNA is paired with a counterion and then subjected to reverse phase chromatography using the nonporous beads as described herein. Aqueous mobile phase containing ion pairing reagent is applied to the column at an initial concentration of organic component that is low enough such that all of the RNA molecules of interest bind to the column. The RNA molecules elute as

the concentration of organic component in the mobile phase is increased. The concentration of organic component preferably is applied as a gradient in order to elute the RNA molecules. The gradient can be a linear gradient, although curved or step gradients can also be used.

In carrying out the segregation according to the present method, the aqueous mobile phase contains a counterion agent and an organic solvent.

Any of a number of mobile phase components typically utilized in ion-pairing reverse phase HPLC are suitable for use in the present invention. Several mobile phase parameters (e.g., pH, organic solvent, counterion, and elution gradient) may be varied to achieve optimal segregation.

The concentration of the mobile phase components will vary depending upon the nature of the segregation to be carried out. The mobile phase composition may vary from sample and during the course of the sample elution. Gradient systems containing two or more components may be used.

Samples are typically eluted by starting with an aqueous or mostly aqueous mobile phase containing a counterion agent and progressing to a mobile phase containing increasing amounts of an organic solvent. Any of a number of gradient profiles and system components may be used to effect the elution. One such exemplary gradient system in accordance with the invention is a linear binary gradient system composed of (i) 0.1 molar triethylammonium acetate and (ii) 25% acetonitrile in a solution of 0.1 molar triethylammonium acetate.

In a preferred embodiment, the elution from the reverse phase column is carried out under conditions effective to denature the secondary structure of the RNA molecules. For example, the elution can be carried out a mobile phase temperature that is high enough to melt all secondary structure of the RNA molecules. For example, the denaturation can be accomplished by conducting the elution at a temperature greater than about 60°C and more preferably above 70°C. An operable temperature is within the range of about 40°C to about 80°C.

Alternatively, denaturation can be effected by including chemical denaturing agents such as urea, formamide, or guanidinum salts in the mobile

phase, or by adjustment of the pH of the mobile phase. The pH of the mobile phase is preferably within the range of about pH 5 to about pH 9, and optimally within the range of about pH 6 to about pH 7.5.

In one preferred embodiment, the pH of the mobile phase is adjusted to effect denaturation of the secondary structure of the RNA molecules. In using chemical means to effect such denaturation, the pH may be adjusted by addition of either base (e.g., sodium hydroxide or urea to a pH of around about 8) or acid (e.g., triethylamine and acetic acid at a pH of about 8) under conditions effective to denature the RNA molecules and which do not degrade the RNA molecules present in the sample nor adversely affect the integrity of the stationary phase.

Fractions eluting from the MIPC system can be collected as a single fraction or as a plurality of fractions. The collection can be performed manually (e.g., from conduit 90) or using an automated fraction collector.

The method of the invention can be used to segregate molecules having lengths exceeding about 100 nt. The method is especially useful in segregating RNA molecules above a length exceeding about 200 nt. The method can also be used in segregating RNA molecules of about 50 to 200 nt from RNA molecules of about 500 to 2,000. The method can be used in segregating RNA molecules having lengths of about 500 to about 1,000 from molecules having lengths of about 3,000 to about 20,000. The method can be used in segregating RNA molecules having lengths within the range of about 2,000 nt to about 20,000 nt. The method can be used to segregate tRNA from rRNA, and can be used to segregate tRNA and rRNA from mRNA molecules which exceed the length of the largest rRNA.

MIPC segregation of RNA has the advantage of providing intact RNA molecules, or size ranges of RNA molecules, and avoiding chemical covalent modification which can occur using prior art gel based separation methods. Also, segregation by MIPC can be accomplished in about 10-30 min, in contrast to conventional gel chromatography which can require hours or days. The shorter processing times required in the present method decrease the chance for RNA degradation to occur.

The present method and system can be used with RNA obtained from a variety of biological samples and extracted using conventional procedures. Such extraction techniques are described in detail in a number of U.S. Patents (e.g., 5,945,515; 5,973,137; 5,438,128) and in standard molecular biology methodology texts, including T. Maniatis et al., "Molecular Cloning. A Laboratory Manual.", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1982) and L.G. Davis et al, "Basic Methods in Molecular Biology", Elsevier, N.Y. (1986).

Particular examples of the segregation of RNA molecules are described in the Examples herein.

In the chromatogram shown in FIG. 15, as described in Example 1, RNA size standards ranging in size from 155 to 1770 nucleotides were segregated using the system and method of the present invention. FIG. 23 is another example of segregation of RNA size standards.

Another example of an RNA segregation is described in Example 2 (FIG. 16) in which a sample of total RNA from a plant source was applied to a reverse phase column of the invention and eluted at 75°C. Peaks were observed for different classes of RNA in order of their respective sizes with tRNA (70-150 nt) eluting first, followed by rRNA (1,500 to 3,700 nt). rRNA, the most abundant species, appeared as two large peaks between about 13-15 min. and had the greatest area under the curve. In order to enrich for mRNA, a portion of the plant total RNA was further purified using a spin column containing polystyrene-latex particles covalently linked to dT oligonucleotides, according to the instructions provided with the column (Catalog no. 70022, Qiagen). Analysis of the resulting product by MIPC (FIG. 17) indicated a decrease in rRNA (appearing at about 14 min) and a relative increase in an mRNA peak (appearing at about 17 min). A further decrease in the rRNA peak was observed after an additional spin column purification (FIG. 18).

Another representative example of an RNA segregation is described in Example 3 (FIG. 22). Equal volume aliquots of mobile phase were collected each minute from 5 to 13 minutes. Further work (data not shown) indicated that fractions 5-8 were relatively enriched for fragments under 2,000 nt. Fractions 9

and 10 were enriched for fragments between about 1,000 and about 15,000 nt. Fraction 11 was enriched for fragments having between about 2,000 and about 10,000 nt.

A further representative example of an RNA segregation is described in Example 4 (FIG. 20) in which a standard mixture of human brain mRNA was analyzed using a 50 mm x 4.6 mm ID column. The heterogeneous mixture showed a single broad peak at about 9.9 min. Applicants surprisingly observed dramatic improvement in segregation of mRNA when the elution was conducted using a column having an ID of 7.8 mm (FIG. 21) as described in Example 5. Additional peaks at about 9.9 min and 10.41 min were observed on the leading edge of the broad peak eluting at 11.8 min. Improved segregation of RNA molecules having lengths exceeding 100 nucleotides pairs was obtained using columns having internal diameters greater than about 5 mm. Improved resolution during the segregation of RNA by MPIC is obtained using a column having a ID of greater than 5 mm, preferably greater than about 7 mm, more preferably greater than about 10 mm. In other embodiments, improved segregation is obtained with a column having an ID within the range of about 5 mm to about 1 m.

In another aspect, the invention concerns a method for stabilizing RNA molecules in a mixture by removing RNases from the mixture. A preferred embodiment of this aspect includes applying a solution of the RNA molecules and counterion reagent to a column containing polymeric beads having non-polar surfaces and flowing mobile phase through the column, the mobile phase having a concentration of organic component less than a concentration necessary to elute the RNA molecules and at which the RNases are not retained on the column.

This aspect of the invention is based in part on Applicant's surprising discovery that RNA molecules that have been eluted from the column are remarkably stable.

Applicants have observed that the rate of degradation is greatly reduced in RNA molecules in samples eluted from the MIPC system. This observation was

investigated in time course experiments in which the stability of RNA was examined under a variety of conditions.

Representative time-course experiments demonstrating the phenomenon of increased stability are described in Examples 10 and 11. For example, in Example 10 a mouse brain mRNA preparation was subjected to MIPC analysis (FIG. 19). Throughout the gradient, aliquots of eluted mobile phase were collected. Each aliquot was divided into three portions thus giving three sets of portions. The three sets were stored over periods ranging up to about 30 days. The first set was stored at room temperature; the second set stored at -20°C; the third set stored at -70°C.

At various time points, each portion in each set was tested to determine whether the mRNA in the portion was intact by testing for intact β-actin mRNA. The test involved subjecting a sampling from each portion to reverse transcription (RT) followed by polymerase chain reaction (PCR) using β-actin specific primers to form a 550 base pair double stranded cDNA fragment. MIPC analysis under non-denaturing conditions was used to determine the quantity of the amplified 550 bp fragment in each portion.

A representative chromatograph of the 550 base pair fragment (derived from the 7 min aliquot) is shown in FIG. 24. The area under the curve for the 550 base pair fragment for each portion was determined using data analysis software.

At each time point, the areas under the curve for the 550 bp fragment for all the portions within a set (e.g., the portions retained at -70°C) were summed in order to quantify the mRNA. The amount of β-actin DNA on day 1 was set to 100%.

The results, showing the amount of remaining β-actin DNA relative to the amount on day 1 are summarized in the Table I, and indicated no degradation of mRNA in portions retained at -20°C and -70°C for up to 23 days. For portions retained at room temperature, there was no degradation of mRNA for up to 7 days, and a loss of about 25% of the mRNA by the 23rd day.

Other time-course studies were conducted to determine the stability of mRNA which had not been subjected to passage through a reverse phase column in a MIPC separation, e.g., Example 12.

In a representative study (described in Example 12), digitoxin-labeled β-actin RNA was obtained commercially and, in a series of test mixtures, a known amount of this RNA was mixed with selected concentrations of acetonitrile (which varied from 0% to 90% acetonitrile in water).

At various time points, the presence of intact mRNA in each test mixture was determined using RT and PCT to obtain a 550 bp β-actin cDNA. The cDNA was quantified as described in Example 10.

It will be appreciated that during an RNA segregation using elution conditions such as described in Example 1 in which the highest value for %B is 70%, the concentration of acetonitrile in any collected aliquots would be no greater than 17.5% (0.25 x 70%). For an aliquot collected at about 7 min, the concentration in the aliquot would be 12% acetonitrile. In Example 10, none of the mixtures have been applied to an MIPC column, but have merely been mixed and stored. Therefore, the mixtures in Example 12 containing less than 20% acetonitrile simulate closely the actual conditions that would exist in an eluted aliquot.

Considering mixtures containing 0%, 5% and 10% acetonitrile, the results (*FIG. 27*) indicated that in 30 days, the mRNA had degraded by a large amount (more than 50%) regardless of the presence of acetonitrile in the starting solutions both for mixtures at room temperature and for those stored at -20°C. Similar experiments, but including the counterion agent TEAA were conducted, and showed the same results as with acetontrile alone.

Without wishing to be bound by theory, one explanation for the observed stabilization phenonmenon is that the RNase enzymes are separated from the RNA molecules so that the eluted RNA is essentially free from contaminating RNase enzymes. This could occur, for example, if RNase was retained on the column, and did not elute, or if the RNase washed through the column, prior to initiating the mobile phase gradient.

In order to more fully understand the observed stabilizing effect, Applicants applied a preparation containing a purified RNase (RNase 1) directly to the column (Example 13) using the same elution conditions as used for segregation of mRNA. The retention time (Rt) for the solvent front was determined by injection of water at t=0 min, and observing a peak at 0.52 min during an MIPC analysis (FIG. 25). As shown in FIG. 26, after injecting RNase, a large peak appeared at the Rt of the solvent front. Upon elution of the column with increasing concentrations of organic component, no further peaks were observed.

Without wishing to be bound by theory, an explanation for the enhanced stability of RNA eluted from the reverse phase chromatographic column as described herein is due to a difference in the ability of the RNase and the RNA molecules to bind to the column. In an injection of RNA molecules, contaminating RNase is eluted at the beginning of the gradient under initial conditions in which the RNA molecules remain retained on the column. The RNA molecules can then be eluted with a mobile phase gradient containing increasing organic component. It was concluded that the 0.52 min peak (FIG. 26) contained essentially all of the injected RNase protein, consistent with an hypothesis that the RNase was not retained by the reverse phase column.

In general, the only requirement for the reverse phase beads of the present invention is that they must have a surface that is either intrinsically non-polar or be bonded with a material that forms a surface having sufficient non-polarity to interact with a counterion agent.

The non-porous polymeric beads can have an average diameter of about 0.5 -100 microns; preferably, 1 - 10 microns; more preferably, 1 - 5 microns. Beads having an average diameter of 1.0 - 3.0 microns are most preferred.

Without wishing to be bound by theory, Applicants believe that the beads which are operable in RNA segregation as described herein have a pore size which essentially excludes the RNA molecules being separated from entering the bead. As used herein, the term "nonporous" is defined to denote a bead which has surface pores having a diameter that is less than the size and shape of the

smallest RNA molecule in the mixture in the solvent medium used therein. Included in this definition are polymer beads having these specified maximum size restrictions in their natural state or which have been treated to reduce their pore size to meet the maximum effective pore size required.

The surface conformations of nonporous beads of the present invention can include depressions and shallow pit-like structures which do not interfere with the segregation process. A pretreatment of a porous bead to render it nonporous can be effected with any material which will fill the pores in the bead structure and which does not significantly interfere with the MIPC process.

Pores are open structures through which mobile phase and other materials can enter the bead structure. Pores are often interconnected so that fluid entering one pore can exit from another pore. Applicants believe that pores having dimensions that allow movement of the RNA into the interconnected pore structure and into the bead impair the segregation of RNA molecules or result in segregations that have very long retention times. In MIPC, however, the preferred beads are "nonporous" and the polynucleotides do not enter the bead structure.

Chromatographic efficiency of the column beads is predominantly influenced by the properties of surface and near-surface areas. For this reason, the following descriptions are related specifically to the close-to-the-surface region of the polymeric beads. The main body and/or the center of such beads can exhibit entirely different chemistries and sets of physical properties from those observed at or near the surface of the polymeric beads of the present invention.

The nonporous polymeric beads of the present invention are prepared by a two-step process in which small seed beads are initially produced by emulsion polymerization of suitable polymerizable monomers. The emulsion polymerization procedure of the invention is a modification of the procedure of Goodwin, et al. (Colloid & Polymer Sci., 252:464-471 (1974)). Monomers which can be used in the emulsion polymerization process to produce the seed beads include styrene, alkyl substituted styrenes, alpha-methyl styrene, and alkyl

substituted alpha-methyl styrene. The seed beads are then enlarged and, optionally, modified by substitution with various groups to produce the nonporous polymeric beads of the present invention.

The seed beads produced by emulsion polymerization can be enlarged by any known process for increasing the size of the polymer beads. For example, polymer beads can be enlarged by the activated swelling process disclosed in U.S. Patent No. 4,563,510. The enlarged or swollen polymer beads are further swollen with a crosslinking polymerizable monomer and a polymerization initiator. Polymerization increases the crosslinking density of the enlarged polymeric bead and reduces the surface porosity of the bead. Suitable crosslinking monomers contain at least two carbon-carbon double bonds capable of polymerization in the presence of an initiator. Preferred crosslinking monomers are divinyl monomers, preferably alkyl and aryl (phenyl, naphthyl, etc.) divinyl monomers and include divinyl benzene, butadiene, etc. Activated swelling of the polymeric seed beads is useful to produce polymer beads having an average diameter ranging from 1 up to about 100 microns.

Alternatively, the polymer seed beads can be enlarged simply by heating the seed latex resulting from emulsion polymerization. This alternative eliminates the need for activated swelling of the seed beads with an activating solvent. Instead, the seed latex is mixed with the crosslinking monomer and polymerization initiator described above, together with or without a water-miscible solvent for the crosslinking monomer. Suitable solvents include acetone, tetrahydrofuran (THF), methanol, and dioxane. The resulting mixture is heated for about 1 - 12 hours, preferably about 4 - 8 hours, at a temperature below the initiation temperature of the polymerization initiator, generally, about 10°C - 80°C, preferably 30°C - 60°C. Optionally, the temperature of the mixture can be increased by 10 - 20% and the mixture heated for an additional 1 to 4 hours. The ratio of monomer to polymerization initiator is at least 100:1, preferably about 100:1 to about 500:1, more preferably about 200:1 in order to ensure a degree of polymerization of at least 200. Beads having this degree of polymerization are sufficiently pressure-stable to be used in high pressure liquid chromatography

(HPLC) applications. This thermal swelling process allows one to increase the size of the bead by about 110 - 160% to obtain polymer beads having an average diameter up to about 5 microns, preferably about 2 - 3 microns. The thermal swelling procedure can, therefore, be used to produce smaller particle sizes previously accessible only by the activated swelling procedure.

Following thermal enlargement, excess crosslinking monomer is removed and the particles are polymerized by exposure to ultraviolet light or heat. Polymerization can be conducted, for example, by heating of the enlarged particles to the activation temperature of the polymerization initiator and continuing polymerization until the desired degree of polymerization has been achieved. Continued heating and polymerization allows one to obtain beads having a degree of polymerization greater than 500.

In the present invention, the packing material disclosed by Bonn et al. or U.S. Patent No. 4,563,510 can be modified through substitution of the polymeric beads with alkyl groups or can be used in its unmodified state. For example, the polymer beads can be alkylated with 1 or 2 carbon atoms by contacting the beads with an alkylating agent, such as methyl iodide or ethyl iodide. Alkylation is achieved by mixing the polymer beads with the alkyl halide in the presence of a Friedel-Crafts catalyst to effect electrophilic aromatic substitution on the aromatic rings at the surface of the polymer blend. Suitable Friedel-Crafts catalysts are well-known in the art and include Lewis acids such as aluminum chloride, boron trifluoride, tin tetrachloride, etc. The beads can be hydrocarbon substituted by substituting the corresponding hydrocarbon halide for methyl iodide in the above procedure, for example.

The term alkyl as used herein in reference to the beads of the present invention is defined to include alkyl and alkyl substituted aryl groups, having from 1 to 1,000,000 carbons, the alkyl groups including straight chained, branch chained, cyclic, saturated, unsaturated nonionic functional groups of various types including aldehyde, ketone, ester, ether, alkyl groups, and the like, and the aryl groups including as monocyclic, bicyclic, and tricyclic aromatic hydrocarbon groups including phenyl, naphthyl, and the like. Methods for alkyl substitution are

conventional and well-known in the art and are not an aspect of this invention. The substitution can also contain hydroxy, cyano, nitro groups, or the like which are considered to be non-polar, reverse phase functional groups.

In the present invention, successful segregation of RNA molecules can be achieved using underivatized nonporous beads as well as using beads derivatized with alkyl groups having 1 to 1,000,000 carbons.

The base polymer of the invention can also be other polymers, non-limiting examples of which include mono- and di-vinyl substituted aromatics such as styrene, substituted styrenes, alpha-substituted styrenes and divinylbenzene; acrylates and methacrylates; polyolefins such as polypropylene and polyethylene; polyesters; polyurethanes; polyamides; polycarbonates; and substituted polymers including fluorosubstituted ethylenes commonly known under the trademark TEFLON. The base polymer can also be mixtures of polymers, non-limiting examples of which include poly(styrene-divinylbenzene) and poly(ethylvinylbenzene-divinylbenzene). Methods for making beads from these polymers are conventional and well known in the art (for example, see U.S. Patent 4,906,378). The physical properties of the surface and near-surface areas of the beads are the predominant influence on chromatographic efficiency. The polymer, whether derivatized or not, preferably provides a nonporous, non-reactive, and non-polar surface for the MIPC segregation.

To achieve optimal results, it is generally necessary to tightly pack the chromatographic column with the solid phase polymer beads. Any known method of packing the column with a column packing material can be used in the present invention to obtain adequate high resolution separations. Typically, a slurry of the polymer beads is prepared using a solvent having a density equal to or less than the density of the polymer beads. The column is then filled with the polymer bead slurry and vibrated or agitated to improve the packing density of the polymer beads in the column. Mechanical vibration or sonication are typically used to improve packing density.

For example, to pack a 50 x 7.8 mm ID column, 3.0 grams of beads can be suspended in 15 mL of methanol with the aid of sonication. The suspension

is then packed into the column using 100 mL of methanol at 8,000 psi pressure. This improves the density of the packed bed.

There are several types of counterions suitable for use with MIPC. These include a mono-, di-, or trialkylamine that can be protonated to form a positive counter charge or a quaternary alkyl substituted amine that already contains a positive counter charge. The alkyl substitutions may be uniform (for example, triethylammonium acetate or tetrapropylammonium acetate) or mixed (for example, propyldiethylammonium acetate). The size of the alkyl group may be small (methyl) or large (up to 30 carbons) especially if only one of the substituted alkyl groups is large and the others are small. For example octyldimethylammonium acetate is a suitable counterion agent. Preferred counterion agents are those containing alkyl groups from the ethyl, propyl or butyl size range.

The purpose of the alkyl group is to impart a nonpolar character to the RNA through a matched ion process so that the RNA can interact with the nonpolar surface of the reverse phase media. The requirements for the extent of nonpolarity of the counterion-RNA pair depends on the polarity of the reverse phase media, the solvent conditions required for RNA segregation, the particular size and type of molecules being segregated. For example, if the polarity of the reverse phase media is increased, then the polarity of the counterion agent may have to change to match the polarity of the surface and increase interaction of the counterion-RNA pair. Triethylammonium acetate is preferred although quaternary ammonium reagents such as tetrapropyl or tetrabutyl ammonium salts can be used when extra nonpolar character is needed or desired.

In the mobile phase of the present method, an organic solvent that is water soluble is preferably used, for example, alcohols, nitriles, dimethylformamide (DMF), tetrahydrofuran (THF), esters, and ethers. Water soluble solvents are defined as those which exist as a single phase with aqueous systems under all conditions of operation of the present invention. Solvents which are particularly preferred for use in the method of this invention include

methanol, ethanol, 2-propanol, 1-propanol, tetrahydrofuran (THF), and acetonitrile, with acetonitrile being most preferred overall.

In some cases, it may be desired to increase the range of concentration of organic solvent used to perform the segregation. For example, increasing the alkyl length on the counterion agent will increase the nonpolarity of the counterion-RNA pair resulting in the need to either increase the concentration of the mobile phase organic component, or increase the strength of the organic component type. There is a positive correlation between concentration of the organic solvent required to elute a fragment from the column and the length of the fragment. However, at high organic solvent concentrations, the RNA could precipitate. To avoid precipitation, a strong organic solvent or a smaller counterion alkyl group can be used. The alkyl group on the counterion reagent can also be substituted with halides, nitro groups, or the like to moderate polarity.

The mobile phase preferably contains a counterion agent. Typical counterion agents include trialkylammonium salts of organic or inorganic acids, such as lower alkyl primary, secondary, and lower tertiary amines, lower trialkyammonium salts and lower quaternary alkyalmmonium salts. Lower alkyl refers to an alkyl radical of one to six carbon atoms, as exemplified by methyl, ethyl, n-butyl, i-butyl, t-butyl, isoamyl, n-pentyl, and isopentyl. Examples of counterion agents include octylammonium acetate, octadimethylammonium acetate, decylammonium acetate, octadecylammonium acetate, pyridiniumammonium acetate, cyclohexylammonium acetate, diethylammonium acetate, propylethylammonium acetate, propyldiethylammonium acetate, butylethylammonium acetate, methylhexylammonium acetate, tetramethylammonium acetate, tetraethylammonium acetate, tetrapropylammonium acetate, tetrabutylammonium acetate, dimethydiethylammonium acetate, triethylammonium acetate, tripropylammonium acetate, tributylammonium acetate, tetrapropylammonium acetate, and tetrabutylammonium acetate. Although the anion in the above examples is acetate, other anions may also be used, including carbonate, phosphate, sulfate, nitrate, propionate, formate, chloride, and bromide, or any combination of cation

and anion. These and other agents are described by Gjerde, et al. in *Ion Chromatography, 2nd Ed.*, Dr. Alfred Hüthig Verlag Heidelberg (1987). Counterion agents that are volatile are preferred for use in the method of the invention, with triethylammonium acetate (TEAA) and triethylammonium hexafluoroisopropyl alcohol being most preferred.

The mobile phase can optionally include a chelating agent at a concentration of about 0.01 to about 1.0 mM. Examples of preferred chelating agents include water soluble chelating agents and crown ethers. Non-limiting examples of multivalent chelating agents which can be used in the present invention include acetylacetone, alizarin, aluminon, chloranilic acid, kojic acid, morin, rhodizonic acid, thionalide, thiourea, α-furildioxime, nioxime, salicylaldoxime, dimethylglyoxime, α-furildioxime, cupferron, α-nitroso-β-naphthol, nitroso-R-salt, diphenylthiocarbazone, diphenylcarbazone, eriochrome black T, PAN, SPADNS, glyoxal-bis(2-hydroxyanil), murexide, α-benzoinoxime, mandelic acid, anthranilic acid, ethylenediamine, glycine, triaminotriethylamine, thionalide, triethylenetetramine, EDTA, metalphthalein, arsonic acids, α,α'-bipyridine, 4-hydroxybenzothiazole, 8-hydroxyquinaldine, 8-hydroxyquinoline, 1,10-phenanthroline, picolinic acid, quinaldic acid, α,α',α''-terpyridyl, 9-methyl-2,3,7-trihydroxy-6-fluorone, pyrocatechol, salicylic acid, tiron, 4-chloro-1,2-dimercaptobenzene, dithiol, mercaptobenzothiazole, rubeanic acid, oxalic acid, sodium diethyldithiocarbarbamate, and zinc dibenzyldithiocarbamate. These and other examples are described by Perrin in *Organic Complexing Reagents: Structure, Behavior, and Application to Inorganic Analysis*, Robert E. Krieger Publishing Co. (1964). In the present invention, a preferred multivalent cation binding agent is EDTA.

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

EXAMPLE 1

RNA Segregation of an RNA Sizing Standard by MIPC using a 7.8 mm ID Column

MIPC analysis of a 0.16-1.77 Kb RNA ladder (Catalog no. 15623010, Life Technologies) was performed using octadecyl modified, nonporous poly(ethylvinylbenzene-divinylbenzene) beads packed in a 50 mm x 7.8 mm ID reverse phase column (DNASEP® cartridge, Transgenomic, Inc., San Jose, CA) and using a WAVE® Nucleic Acid Fragment Analysis System (Transgenomic). Buffer A: 0.1 M TEAA, pH 7.0; buffer B: 0.1 TEAA, 25% (v/v) acetonitrile, pH 7.0. The gradient conditions were as follows:

Time (min)	%B
0.0	38
1.0	40
16	60
22	66
22.5	70
23	100
24	100
25	38
27	38

The flow rate was 0.9 mL/min and the column temperature was 75.0°C. UV detection was performed at 260nm. The injection volume was 5.0 μL. The sample contained a mixture of eight RNAs having the nucleotide lengths as shown in FIG. 15.

Prior to the injection, the column was equilibrated with 75% acetonitrile for 30-45 min at a flow rate of 0.9 mL/min. The column was then equilibrated using 38%B for 30 min. Prior to the elution of RNA, two control gradient elutions (using the same gradient conditions as for the RNA) were performed: a first injection of 10μL of 0.5 mM EDTA and a second injection of 10μL of nuclease free water

(Catalog no. 9930, Ambion, Inc., Austin, TX). These two injections (data not shown) demonstrated that the column was free from contamination.

Another sizing standard (catalog no. 1062611, Roche Molecular Biochemicals, Indianapolis, IN) was similarly analyzed as shown in FIG. 23. 1μg RNA was injected in a volume of 1μL.

In preparing the mRNA sample for injection, all chemicals were of the highest purity grade available for molecular biology. Solutions, glassware, and small instruments were sterilized whenever possible. Liquid transfers were made using RNase free pipette tips (Rainin Instrument Co., Inc., Woburn, MA). All manipulations were performed wearing surgical gloves.

EXAMPLE 2

RNA Segregation of Tobacco Plant RNA by MIPC using a 7.8 mm ID Column

Total RNA was extracted from the flower of tobacco plant (Nicotiana *tabacum* cv. Wisconsin 38) by an acid guanidinium thiocyanate phenol-chloroform extraction method, and precipitated with 4 M lithium chloride (Chomczynski, et al., Anal. Biochem. 162:156-159 (1987) as described in Bahrami, et al., Plant Molecular Biology, 39:325-333 (1999).

MIPC analysis of total RNA from the plant extract was performed using octadecyl modified, nonporous poly(ethylvinylbenzene-divinylbenzene) beads packed in a 50 mm x 7.8 mm ID reverse phase column (DNASEP® cartridge, Transgenomic, Inc., San Jose, CA) and using a WAVE® Nucleic Acid Fragment Analysis System (Transgenomic). Buffer A: 0.1 M TEAA, pH 7.0; buffer B: 0.1 TEAA, 25% (v/v) acetonitrile, pH 7.0. The gradient conditions were as described in Example 1. The volume injected was 2 μL (containing 1.54μg RNA). The chromatogram is shown in FIG. 16.

EXAMPLE 3

RNA Segregation of Mouse Brain mRNA by MIPC using a 7.8 mm ID Column

MIPC analysis was performed using mouse brain mRNA and elution conditions as described in Example 1. For FIG. 19, the source of the mRNA was Clontech (catalog no. 7813).

In FIG. 22, a different preparation of mouse brain mRNA was used and 5μg RNA was injected in a volume of 3 μL. Fractions of mobile phase (0.9 mL each) were collected each minute from 5 to 13 minutes. Further work, including reverse transcription and conventional gel analysis (not shown), indicated that fractions collected at 5-8 min were enriched for fragments under 2,000 nt. Fractions collected at 9 and at 10 min were enriched for fragments between about 1,000 and about 15,000 nt. The fraction collected at 11 min was enriched for fragments having between about 2,000 and about 10,000 nt.

EXAMPLE 4

RNA Segregation of Human Brain mRNA by MIPC using a 4.6 mm ID Column

MIPC analysis of human brain mRNA (Catalog no. 6516-1, Clontech Laboratories, Inc., Palo Alto, CA) was performed using octadecyl modified, nonporous poly(ethylvinylbenzene-divinylbenzene) beads packed in a 50 mm x 4.6 mm ID reverse phase column (DNASEP® cartridge, Transgenomic, Inc., San Jose, CA) and using a WAVE® Nucleic Acid Fragment Analysis System (Transgenomic). Buffer A: 0.1 M TEAA, pH 7.0; buffer B: 0.1 TEAA, 25% (v/v) acetonitrile, pH 7.0. The gradient conditions were as follows:

Time (min)	%B
1.0	38
1.0	40
16	60
22	66
22.5	70

The flow rate was 0.9 mL/min and the column temperature was 75.0°C. UV detection was performed at 260nm. Injection volume was 4.5μL. The chromatogram is shown in FIG. 20.

EXAMPLE 5

RNA Segregation of Human Brain mRNA by MIPC using a 7.8 mm ID Column

MIPC analysis was performed using the same mRNA sample and conditions as described in Example 4 except that the column was replaced by a

50 mm x 7.8 mm ID column. The injection volume was 5.5μL. The chromatogram is shown in FIG. 21.

EXAMPLE 6

Preparation of nonporous poly(styrene-divinylbenzene) particles

Sodium chloride (0.236 g) was added to 354 mL of deionized water in a reactor having a volume of 1.0 liter. The reactor was equipped with a mechanical stirrer, reflux condenser, and a gas introduction tube. The dissolution of the sodium chloride was carried out under inert atmosphere (argon), assisted by stirring (350 rpm), and at an elevated temperature (87^0C). Freshly distilled styrene (33.7 g) and 0.2184 g of potassium peroxodisulfate ($K_2S_2O_8$) dissolved in 50 mL of deionized water were then added. Immediately after these additions, the gas introduction tube was pulled out of the solution and positioned above the liquid surface. The reaction mixture was subsequently stirred for 6.5 hours at 87^0C. After this, the contents of the reactor were cooled down to ambient temperature and diluted to a volume yielding a concentration of 54.6 g of polymerized styrene in 1000 mL volume of suspension resulting from the first step. The amount of polymerized styrene in 1000 mL was calculated to include the quantity of the polymer still sticking to the mechanical stirrer (approximately 5 - 10 g). The diameter of the spherical beads in the suspension was determined by light microscopy to be about 1.0 micron.

Beads resulting from the first step are still generally too small and too soft (low pressure stability) for use as chromatographic packings. The softness of these beads is caused by an insufficient degree of crosslinking. In a second step, the beads are enlarged and the degree of crosslinking is increased.

The protocol for the second step is based on the activated swelling method described by Ugelstad et al. (*Adv. Colloid Interface Sci.*, 13:101-140 (1980)). In order to initiate activated swelling, or the second synthetic step, the aqueous suspension of polystyrene seeds (200 ml) from the first step was mixed first with 60 mL of acetone and then with 60 mL of a 1-chlorododecane emulsion. To prepare the emulsion, 0.206 g of sodium dodecylsulfate, 49.5 mL of deionized water, and 10.5 mL of 1-chlorododecane were brought together and the resulting

mixture was kept at 0^0C for 4 hours and mixed by sonication during the entire time period until a fine emulsion of < 0.3 microns was obtained. The mixture of polystyrene seeds, acetone, and 1-chlorododecane emulsion was stirred for about 12 hours at room temperature, during which time the swelling of the beads occurred. Subsequently, the acetone was removed by a 30 minute distillation at 80°C.

Following the removal of acetone, the swollen beads were further grown by the addition of 310 g of a ethyldivinylbenzene and divinylbenzene (DVB) (1:1.71) mixture also containing 2.5 g of dibenzoylperoxide as an initiator. The growing occurred with stirring and with occasional particle size measurements by means of light microscopy.

After completion of the swelling and growing stages, the reaction mixture was transferred into a separation funnel. In an unstirred solution, the excess amount of the monomer separated from the layer containing the suspension of the polymeric beads and could thus be easily removed. The remaining suspension of beads was returned to the reactor and subjected to a stepwise increase in temperature (63°C for about 7 hours, 73°C for about 2 hours, and 83°C for about 12 hours), leading to further increases in the degree of polymerization (> 500). The pore size of beads prepared in this manner was below the detection limit of mercury porosimetry (< 30Å).

After drying, the dried beads (10 g) from step two were washed four times with 100 mL of n-heptane, and then two times with each of the following: 100 mL of diethylether, 100 mL of dioxane, and 100 mL of methanol. Finally, the beads were dried.

EXAMPLE 7

Alkylation of Poly(Styrene-Divinylbenzene) Polymer Beads

The following procedures were carried out under nitrogen (Air Products, Ultra Pure grade, Allentown, PA) at a flow rate of 250-300 mL/min. 25 g of the beads prepared in Example 5 were suspended in 150-160 g of 1-chlorooctadecane (product no. 0235, TCI America, Portland, OR) using a bow shaped mixer (use a 250 mL wide neck Erlenmeyer flask). The temperature was

set to 50-60°C to prevent the 1-chlorooctadecane from solidifying. Larger pieces of polymer were broken up to facilitate suspending. The solution was mixed using a stirrer (Model RZRI, Caframo, ONT NOH2T0, Canada) with the speed set at 2. The polymer suspension was transferred into a three neck bottle (with reflux condenser, overhead stirrer and gas inlet). 52-62 g of 1-chlorooctadecane were used to rinse the Erlenmeyer flask and were added to the three neck bottle. The bottle was heated in an ethylene glycol bath set at 80°C. The solution was mixed using a stirrer (Caframo) with the speed set at 0. After 20 minutes, the reaction was started by addition of 1.1 g $AlCl_3$ powder (product no. 06218, Fluka, Milwaukee, WI) and continued for 16-18 h.

After the reaction, the polymer was separated from excess 1-chlorooctadecane by centrifugation followed by consecutive washing steps:

Addition	Comment
50 mL conc. HCl, 50-60 mL n-heptane	4 repetitions, with recycled heptane
100 mL H_2O, 50-60 mL n-heptane	1 repetition, with fresh heptane
50 mL conc. HCl, 50-60 mL n-heptane	1 repetition, with fresh heptane
100 mL H_2O, 50-60 mL n-heptane	1 repetition, fresh heptane
150 mL H_2O, no n-heptane	3 repetitions, use plastic stirrer to break up chuncks of polymer beads. Repeat steps 4 and 5 three times. Shake for two minutes with no centrifugation.
100 mL THF	3 repetitions
100 mL THF/ n-heptane	1 repetition
100 mL n-heptane	1 repetition
100 mL THF	1 repetition
100 mL CH_3OH	4 repetitions

In the steps where aqueous solvents (HCl or H_2O) were used, the polymer was shaken for 30 seconds with the aqueous phase before adding n-heptane. n-Heptane was then added and the mixture was shaken vigorously for 2 min. After the final polymeric beads were dried at 40-50°C for 2-3 hr, they were ready for packing.

EXAMPLE 8

Acid Wash Treatment

The beads prepared in Example 7 were washed three times with tetrahydrofuran and two times with methanol. Finally the beads were stirred in a mixture containing 100 mL tetrahydrofuran and 100 mL concentrated hydrochloric acid for 12 hours. After this acid treatment, the polymer beads were washed with a tetrahydrofuran/water mixture until neutral (pH = 7). The beads were then dried at 40°C for 12 hours.

EXAMPLE 9

Column Packing Procedure

After weighing out 3 grams of oven dried polymeric beads, a slurry was formed with 10 mL tetrahydrofuran (THF) and place in a sonicator under a fume hood for 15 min. 5 mL of THF and 5 mL of methanol (MeOH) were added followed by sonication for an additional 10 min. A packing assembly was pre-filled with 20 mL MeOH. The slurry was slowly poured into the packing assembly. A Haskel pump (Haskel International, Inc., Burbank, CA) was turned on and the packing pressure was slowly increased to 5000 psi for the initial packing phase. After 10 min, the packing pressure was slowly increased to 9000 psi and the secondary packing phase set for 20 min. After 20 min, the packing eluent was changed from MeOH to 0.05 M Na_4EDTA. The final packing phase was set for 40 min.

EXAMPLE 10

Assessment of RNA Stabilization by MIPC

Time-course experiments were carried out to demonstrate the phenomenon of increased stability. 5 μL (4.5μg RNA) of mouse brain mRNA (catalog 7813, Ambion) was subjected to MIPC analysis, using acetonitrile as organic component, with the resulting chromatograph shown in FIG. 19. The mobile phase was collected at each minute in 0.9 mL aliquots throughout the acetonitrile gradient.

Each aliquot was divided into three portions so that there were three sets of 0.3 mL liquid samples. The sets were stored in RNase free tubes (Ambion)

over a period ranging up to about 30 days. The first set was stored at room temperature; the second set stored at -20°C; the third set stored at -70°C.

At various time points, each aliquot in each set was tested to determine whether the mRNA in the aliquot was intact. A representative mRNA species, β-actin mRNA, was analyzed in order to test the integrity of mRNA. The test involved subjecting a 5 μL sample from each aliquot to reverse transcription (RT) followed by polymerase chain reaction (PCR) using β-actin specific primers to form a 550 base pair double stranded DNA fragment.

RT-PCR was performed using the TITAN(TM) One Tube RT-PCR Kit (Catalog no. 1939823, Boehringer Mannheim GmbH) according to the directions provided with the kit. The 5 μL from each eluted aliquot was used in a RT-PCR reaction (50 μL final vol.) to generate the β-actin 550bp cDNA fragment. The final concentration of β-actin RNA in each RT-PCR reaction was about 1ng/μL.

PCR was performed using a Perkin Elmer GeneAmp 9600 Thermocycler and using the temperature program shown below:

Cycles	Temperature and time
1x	denature at 50oC for 30 min
1x	denature at 94oC for 2 min
30x	denature at 94oC for 30 sec annealing at 55oC for 30 sec elongation at 68oC for 1 min
1x	prolonged elongation time at 68oC for 7 min

The β-actin specific primers were:

5'-GTCGACAACGGCTCCGGCATG

5'-GGATCTTCATGAGGTAGTCAG

MIPC analysis was used to determine the quantity of the amplified 550 bp fragment in each collected aliquot. A representative chromatograph, derived from the 7 min aliquot, is shown in FIG. 24. The separation was carried out using a

MIPC system (Transgenomic) and a DNASEP column (50mm x 7.8mm ID). The buffers were as indicated in Example 1. The column temperature was 50.0°C. The elution conditions were as follows:

Time (min)	%B
0	56
0.5	61
5.0	70

The 550 base pair DNA fragment appeared at 4.2 min. The area under the curve for the fragment was determined using data analysis software (Hitachi D-7000 HPLC System Manager software).

At each time point, the areas under the curve for the 550 bp fragment for all the portions within a set (e.g., the portions retained at -70°C) were summed in order to quantify the mRNA. The amount of β-actin DNA on day 1 was set to 100%.

The results, showing the amount of remaining β-actin DNA relative to the amount on day 1 are summarized in the Table I, and indicated no degradation of mRNA in portions retained at -20°C and -70°C for up to 23 days. For portions retained at room temperature, there was no degradation of mRNA for up to 7 days, and a loss of about 25% of the mRNA by the 23rd day

Table 1

	Day 0	Day 2	Day 7	Day 23	Day 60
At RT	100%	100%	100%	77%	5%
At –20°C	100%			100%	17%
At –70°C	100%			100%	14%

Example 11

Assessment of RNA stabilization by MIPC

The ability of MIPC fractionation to stabilize RNA was assessed by comparing the stability of fractionated RNA vs. non-fractionated RNA. A mouse brain mRNA sample (Clontech Inc., Palo Alto, CA) was separated by MIPC on a DNASep chromatography cartridge (7.8 mm internal diameter and 50 mm length; Transgenomic, Inc.) using a WAVE Nucleic Acid Fragment Analysis System (Transgenomic, Inc., San Jose, CA). The stationary phase of the cartridge comprises a nonporous, C-18 alkylated poly(styrene-divinylbenzene) separation medium.

Chromatography was performed using a two-eluant buffer system. All eluants and buffers were made using DEPC-treated, double-distilled deionized water, and precautions were taken to maintain a chromatography environment free of metal ions that could interfere with RNA separation. Buffer A consists of an aqueous solution of 0.1 M triethylammonium acetate (TEAA) (pH 7.0) and Buffer B consists of an aqueous solution of 0.1 M TEAA (pH 7.0) with 25% (v/v) of acetonitrile (ACN) (TEAA provided by Transgenomic, Inc., San Jose, CA). The separation was performed under fully denaturing conditions at 75°C. Chromatograms were recorded at a wavelength of 260 nm. The following gradient was used: flow rate 0.9 mL/min, 38 to 40% B in 1.0 min, to 60% B in 15 min, to 66% B in 6.0 min, to 70% B in 0.5 min, to 100 % B in 0.5 min, hold at 100% B for 1 min, to 38% B in 1 min, hold at 38 % B for 2 min.

One minute fractions were collected, precipitated, and reconstituted in 5 µl of DEPC-treated water. Precipitation of eluted RNA samples was performed by addition of 10 % (v/v) of precipitation buffer (10 mM Tris-HCl (pH 7.0), 1 mM EDTA, 3.0 M NaCl), 1 % (v/v) of glycogen (10 mg/mL), and 2.5 volumes of ethanol. Samples were kept at -70 °C for 10 min or at -20 °C for two hours before centrifugation at 13,000 g for 15 min at 4 °C. All precipitated RNA fractions were reconstituted in DEPC-treated water.

RNA stability was assessed by β-actin gene specific RT-PCR on both fractionated (6-15 minute fractions were used) and non-fractionated mouse brain

mRNA samples. Non-fractionated mRNA and reconstituted RNA fractions were reverse transcribed and amplified by RT-PCR using β-actin gene specific primers obtained from Clontech.

In these experiments, the amount of the β-actin RT-PCR product produced from the RNA samples on day zero is set to 100%. Quantification was achieved by MIPC of the RT-PCR product and integration of the area under the chromatogram peak corresponding to the reverse transcript of interest. MIPC and detection was performed as described above. A conversion factor, which is a function of flow rate, was used based on the relationship between peak area and a known amount of DNA analyzed.

The RT-PCR was repeated at different times on stored samples (stored at either room temperature (RT) or at –20°C) and the amount of the β-actin produced was compared to that of day zero, as shown in Tables 1A and 1B. While MIPC fractionated RNA samples kept at room temperature for a period of a month showed less than 25 % reduction in the amount of RT-PCR product, non-fractionated RNA samples kept under the same conditions were completely degraded.

Table 2A

% β-actin produced	Day 0	One month
At RT	100%	77%
At –20	100%	100%

Table 2B

% b-actin produced	Day 0	One month
At RT	100%	0%
At –20	100%	14%

EXAMPLE 12

Stability of mRNA in Solution

Digitoxin-labeled β-actin RNA was obtained commercially (0.01ug RNA/μL, catalog no. 1498-045, Boehringer Mannheim) and, in a series of test mixtures, was mixed (final concentration of 1ng RNA/μL) with selected concentrations of acetonitrile (which varied from 0% to 90% acetonitrile in RNase-free water).

At various time points, the presence of intact mRNA in each test mixture was determined using RT and PCR to obtain a 550 bp β-actin cDNA. The cDNA was quantified as described in Example 10.

It will be appreciated that during an RNA segregation using elution conditions such as described in Example 1 in which the highest value for %B is 70%, the concentration of acetonitrile in any collected aliquots would be no greater than 17.5% (0.25 x 70%). For an aliquot collected at about 7 min, the concentration in the aliquot would be 12% acetonitrile. In Example 10, none of the mixtures have been applied to an MIPC column, but have merely been mixed and stored. Therefore, the mixtures in this example containing less than 20% acetonitrile simulate closely the actual conditions that would exist in an eluted aliquot.

Considering mixtures containing 0%, 5% and 10% acetonitrile, the results (FIG. 27) indicated that in 30 days, the mRNA had degraded by a large amount (more than 50%) regardless of the presence of acetonitrile in the starting solutions both for mixtures at room temperature and for those stored at -20°C. Similar experiments, but including the counterion agent TEAA were conducted, and showed the same results as with acetonitrile alone.

EXAMPLE 13

MIPC Analysis of Purified RNase 1

In order to more fully understand the observed stabilizing effect, 45μL (10 μg protein) of a preparation containing a purified RNase (RNase1 from *E. coli*,

catalog no. M4261/5, Promega Corp. Madison, WI) was injected and analyzed using the gradient conditions described in Example 1.

As shown in FIG. 26 a large peak appeared early after the injection at the retention time (Rt) of the solvent front (0.52 min). The Rt for the solvent front (wash through) was determined by injecting water (10 μL) under the same gradient conditions (FIG. 25). Upon elution of the column with increasing concentrations of organic component, no further peaks were observed.

While the foregoing has presented specific embodiments of the present invention, it is to be understood that these embodiments have been presented by way of example only. It is expected that others will perceive and practice variations which, though differing from the foregoing, do not depart from the spirit and scope of the invention as described and claimed herein.

triethylammonium hexafluoroisopropyl alcohol, and mixtures of one or more thereof.

24. The method of Claim 23 wherein the counterion agent is tetrabutylammonium bromide.

25. The method of Claim 23 wherein the counterion agent is triethylammonium acetate.

26. The method of Claim 1 wherein the RNA molecule is separated from the agent capable of catalyzing RNA degradation by MIPC, wherein mRNA denaturation is achieved by conducting the separation at a temperature sufficient to substantially denature the mRNA molecule, wherein the separation medium comprises polymer beads having an average diameter of 0.5 to 100 microns, and wherein the mobile phase comprises acetonitrile and triethylammonium acetate.

27. The method of Claim 26 wherein the separation is conducted under conditions that are substantially free of multivalent cations capable of interfering with polynucleotide separations.

28. The method of Claim 27, wherein the separation is conducted at a temperature of about 70°C or greater.

29. The method of Claim 28, wherein the separation medium comprises C-18 alkylated nonporous poly(styrene-divinylbenzene) polymer beads.

30. A stabilized RNA molecule prepared by the process recited in Claim 1.

31. A stabilized solution of RNA molecules that is substantially free of RNases.

32. A stabilized solution of RNA molecules that is devoid of RNAse inhibitorsand stable at room temperature.

33. A method for stabilizing an RNA molecule against degradation comprising:

a) applying the RNA molecule to a separation medium having a non-polar separation surface in the presence of a counterion agent;

b) eluting the RNA molecule from the separation medium by passing through the separation medium a mobile phase containing a

concentration of organic solvent sufficient to elute the RNA molecule from the separation medium; and

c) collecting an eluant fraction containing the RNA molecule, wherein the RNA molecule is stabilized against degradation.

17. The method of Claim 1 wherein the separation medium has been prepared using reagents that are substantially free of multivalent cations capable of interfering with polynucleotide separations and under conditions that are substantially free of multivalent cations capable of interfering with polynucleotide separations.

18. The method of Claim 1 wherein the separation medium has been subjected to acid wash treatment to remove any residual surface metal contaminants.

19. The method of Claim 1 wherein the separation medium has been subjected to treatment with a multivalent cation-binding agent.

20. The method of Claim 1 wherein the mobile phase includes an organic solvent selected from the group consisting of alcohol, nitrile, dimethylformamide, tetrahydrofuran, ester, ether, and mixtures of one or more thereof.

21. The method of Claim 20 wherein the mobile phase includes acetonitrile.

22. The method of Claim 1 wherein the mobile phase includes a counterion agent selected from the group consisting of lower alkyl primary amine, lower alkyl secondary amine, lower alkyl tertiary amine, lower trialkylammonium salt, quaternary ammonium salt, and mixtures of one or more thereof.

23. The method of Claim 22 wherein the counterion agent is selected from the group consisting of octylammonium acetate, octadimethylammonium acetate, decylammonium acetate, octadecylammonium acetate, pyridiniumammonium acetate, cyclohexylammonium acetate, diethylammonium acetate, propylethylammonium acetate, propyldiethylammonium acetate, butylethylammonium acetate, methylhexylammonium acetate, tetramethylammonium acetate, tetraethylammonium acetate, tetrapropylammonium acetate, tetrabutylammonium acetate, dimethydiethylammonium acetate, triethylammonium acetate, tripropylammonium acetate, tributylammonium acetate, tetrapropylammonium acetate, tetrabutylammonium acetate,

8. The method of Claim 7 wherein the RNA molecule is separated from the agent capable of catalyzing RNA degradation at a temperature of about 50°C or greater.

9. The method of Claim 8 wherein the RNA molecule is separated from the agent capable of catalyzing RNA degradation at a temperature of about 70°C or greater.

10. The method of Claim 7 wherein the mRNA molecule is substantially denatured by means of a chemical reagent.

11. The method of Claim 1 wherein the separation is conducted under conditions that are substantially free of multivalent cations capable of interfering with polynucleotide separations.

12. The method of Claim 1 wherein the separation medium comprises particles selected from the group consisting of silica, silica carbide, silica nitrite, titanium oxide, aluminum oxide, zirconium oxide, carbon, insoluble polysaccharide, and diatomaceous earth, the particles having separation surfaces which are coated with a hydrocarbon or non-polar hydrocarbon substituted polymer, or have substantially all polar groups reacted with a non-polar hydrocarbon or substituted hydrocarbon group, wherein the surfaces are non-polar.

13. The method of Claim 1 wherein the separation medium comprises polymer beads having an average diameter of 0.5 to 100 microns, the beads being unsubstituted polymer beads or polymer beads substituted with a moiety selected from the group consisting of hydrocarbon having from one to 1,000,000 carbons.

14. The method of Claim 13, wherein the separation medium comprises C-18 alkylated nonporous poly(styrene-divinylbenzene) polymer beads.

15. The method of Claim 1, wherein the separation medium comprises a monolith.

16. The method of Claim 1, wherein the separation medium is substantially free of multivalent cations capable of interfering with polynucleotide separations.